

# PROTEASE STIMULATION BY FOODS IN ADULT *Aedes Aegypti* LINN.<sup>1</sup>

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Much time and money have been devoted to the study of malaria and its vectors. However, most of the research and financial support for it has gone into fields other than the physiology of the various parasites, especially pertaining to their infectivity of mosquitoes. According to Huff (1941 a), "Hardly any other phase of malariology has been so badly neglected as the study of all factors influencing infection in the mosquito."

Of the relatively small amount of data dealing with the malarial parasite infectivity in mosquitoes, a large part of it deals with studies of strains of mosquitoes resistant or susceptible to strains of malarial parasites.

This particular study was undertaken to extend the small field of knowledge of effect of various foods on digestive enzyme elaboration. Since the work of Schlottke (1937 a, b, c), there has been only the work of Day and Powning (1949), Fisk (1950), and Fisk and Shambaugh (1952) studying the stimulatory effects of food on enzyme elaboration. Such research serves to fill in many gaps in the knowledge of the physiology in adult mosquitoes, a physiology not well understood yet extremely important in all considerations of malarial infectivity in mosquitoes.

## METHODS

*Aedes aegypti* Linn. were selected for this study because of the relative ease with which great numbers can be reared. They were obtained from the Research Foundation Project in the Department of Zoology and Entomology sponsored by the United States Public Health Service, which maintains an active colony for its own use.

Female mosquitoes four to six days old were used, for they feed most readily at that age and by that time have disposed of any larval food which remained in the digestive tract at emergence (Fisk, 1950). Four to six days after emergence the mosquitoes were placed in a lamp chimney covered at the top with cheesecloth.

**Feeding and Dissection Techniques.** Mosquitoes were fed either from the bare forearm placed on the cheesecloth on the top of the lamp chimney or through an artificial membrane in a heated feeding apparatus to be described later. The mosquitoes were attracted to the arm or warmed membrane and fed through the gauze. After each one had engorged partially with blood or a blood substitute solution, she was withdrawn from below with an aspirator which was inserted into the chimney through an opening in a cross-slitted rubber dental dam stretched over an embroidery hoop. Each female was placed in a separate vial which was stoppered with a wad of dampened cotton. The fed mosquitoes were placed in an incubator at 30° C. After 18 hr. for digestion, the midguts were dissected from the mosquitoes by the technique outlined by Fisk and Shambaugh (1952).

**Individual Mosquito Weighing.** In this laboratory, as similarly reported by Roy (1936), the range of weight of mosquitoes at a constant age is greater than the average weight for an individual. In order to determine accurately the amount

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of blood ingested by a female mosquito during feeding, it was necessary to obtain individual weights.

A small, rectangular, plastic container was constructed (3.6 cc.) to hold the mosquitoes while weighing them on a Roller-Smith Precision Balance (spring torsion). The mosquitoes were anesthetized with carbon dioxide before weighing. With an aspirator they were transferred to marked shell vials and after revival were allowed to feed on the bare forearm. The amount of blood fed was regulated by disturbing the insect during feeding. Each mosquito was reweighed soon after feeding in the manner just described. The mosquitoes were returned to the marked vials which were stoppered with a wad of damp cotton and placed in an incubator at 30° C for eighteen hours.

*Artificial Feeding Apparatus.* It was necessary to construct an apparatus to keep artificial blood solutions at a constant temperature, 40° C. An "oven" was constructed as follows. Two sizes of Lucite plastic tubing were used: 1) outside diameter 4 cm., inside diameter 3.5 cm., length 3 cm. and 2) outside diameter 5 cm., inside diameter 4.4 cm., length 5 cm. The edges of the tubing were ground flat and smooth with powdered carborundum and alumina. The smaller tube was covered at one end with Baudruche Membrane held in place with a rubber band. When inverted this acted as a reservoir for the solutions. Nichrome resistance wire was wound around the outside of the larger tube and soldered to regular 110 volt copper extension wire. The wires were held in place with an outer insulating layer of furnace cement about one centimeter thick. The smaller tube containing the solutions was placed within the larger tube. This apparatus is a modification of that described by Greenberg (1949).

A cover was prepared for the larger tube to prevent excessive heat loss. A thin, circular, plastic disc 6.3 cm. in diameter was joined with acetone to a plastic ring 4.3 cm. in diameter and 1.5 cm. wide. The heating element was joined in series with a variable rheostat and a 100 watt incandescent bulb. It was necessary to vary the rheostat manually since there was no thermostat in the circuit.

The assembled feeding apparatus was placed on the cheesecloth on the top of the lamp chimney containing mosquitoes. The capping membrane of the inner tube, wet with distilled water, rested on the cheesecloth. Accurate readings of the temperature at the membrane surface were taken with a Leeds and Northrup single range potentiometer connected to a copper-constantan thermocouple placed between the cheesecloth and the capping membrane.

*Preparation of Blood Substitute Solutions.* In *Aedes aegypti*, ingested blood is taken directly into the midgut or rarely the diverticula. Sugar solutions without erythrocytes, in practically all instances, were taken into the diverticula (Trembley, 1952). Most protease activity occurs in the midgut after feeding (Fisk and Shambaugh, 1952). In view of these facts, it was decided to use sheep erythrocytes with substitute solutions so that solutions would be primarily dispatched to the midgut. Fresh sheep blood was defibrinated by mixing 10 percent of its volume with 3 percent sodium citrate. Whole sheep blood was centrifuged 10 min. at 5500 rpm. The supernatant serum was siphoned off. The remaining erythrocytes were diluted to the original volume with physiological saline (0.85% sodium chloride) and stirred before recentrifuging. The washing procedure was repeated twice more. The subsequent thrice-washed erythrocytes were used in all tests requiring erythrocytes.

It was decided to test for any response in protease activity to various fractions of whole blood and to plasma protein fractions. The following solutions were offered: whole human blood, whole sheep blood, washed erythrocytes (cells)+saline, erythrocytes+gum acacia, erythrocytes+serum dialyzate, erythrocytes+serum proteins (non-dialyzable), erythrocytes+0.6 percent fibrinogen, erythrocytes+0.6 percent albumin, erythrocytes+0.6 percent gamma-globulin. The plasma protein fractions were bovine. All of these mixtures were used in a 1:1 volume ratio,

except the solution of erythrocytes+albumin+gamma-globulin+fibrinogen which was in the ratio of 3:1:1:1.

Several techniques were tried to obtain a protein-free plasma but the most useful was dialysis. Ten milliliters of sheep blood were placed in Visking tubing. It was dialyzed in 30 ml. phosphate buffer, pH 7.8, for 3 hr. in an Aminco Mechanical Dialyzer. The dialyzate gave a negative test for proteins upon the addition of trichloroacetic acid. Plasma protein fractions were dissolved in distilled water. The solutions of fibrinogen contained between 40 and 50 percent sodium citrate to prevent its coagulation upon the addition of erythrocytes.

*Injection Technique.* A series of unfed mosquitoes was injected with blood from from 18-hour fed mosquitoes mixed with saline. The injection technique recently described (Shambaugh, 1952) was modified by injecting the saline into the fed mosquito just prior to dissection eighteen hours after feeding. In four instances the midgut contents were mixed with saline and injected into unfed mosquitoes.

*Enzyme Analyses.* The theory and technique for enzyme analyses of midgut breis are discussed adequately in a previous article (Fisk and Shambaugh, 1952). This procedure was used for protease determinations of dissected midguts in lots of 10 or 20. A modified small sample technique was used for less than 10 midguts in a dissection. One milliliter of buffer was added to the homogenate of midguts and the total was brought up to 2 ml. with mosquito saline. The remainder of the technique was unmodified.

#### RESULTS

The conclusions of Fisk and Shambaugh (1952) on the stimulation of proteolytic digestive enzymes after a blood meal as opposed to a meal on a sugar solution served as a basis for further studies of the effect of various blood fractions on protease elaboration in the midgut. The amount of protease activity

TABLE 1

*Summary of data on protease activity of Aedes aegypti females  
eighteen hours after feeding on various food materials.*

FOOD MATERIAL	AVERAGE ACTIVITY PER MIDGUT (Optical Density)
Starved	0.049
Cells + saline	0.144
Cells + 6% gum acacia	0.120
Cells + serum dialyzate	0.216
Cells + serum proteins	0.598
Cells + 0.6% fibrinogen	0.217
Cells + 0.6% albumin	0.256
Cells + 0.6% gamma-globulin	0.338
Cells + albumin + fibrinogen + gamma-globulin	0.377
Whole human blood	0.503

determined was the amount of available protease at the time of dissection, minus the losses due to technique. The amount or condition of the blood meal within the midgut at the time of dissection had no effect on this technique, for the colored materials would be equal in both blanks and runs while the blood protein would be removed by the trichloroacetic acid precipitation.

In the following tables and figures, protease activity will be expressed in terms of optical density of the final solution described in the previous section. Optical density is a direct function of protease activity, being derived from the readings of

the colorimeter by multiplying them by the constant 0.002 (Hawk, et al., 1947). In these tables the number of midguts in the final solution read in the colorimeter depends on the initial number of insects dissected and the technique followed.

*Results With Various Blood Fractions.* Since the peak of digestive enzyme activity was shown by Fisk and Shambaugh (1952) to be 18 hr. after feeding, it was decided to run the experiments under these conditions (see table 1). Starved, or unfed individuals, were used as a check. Thrice-washed erythrocytes were

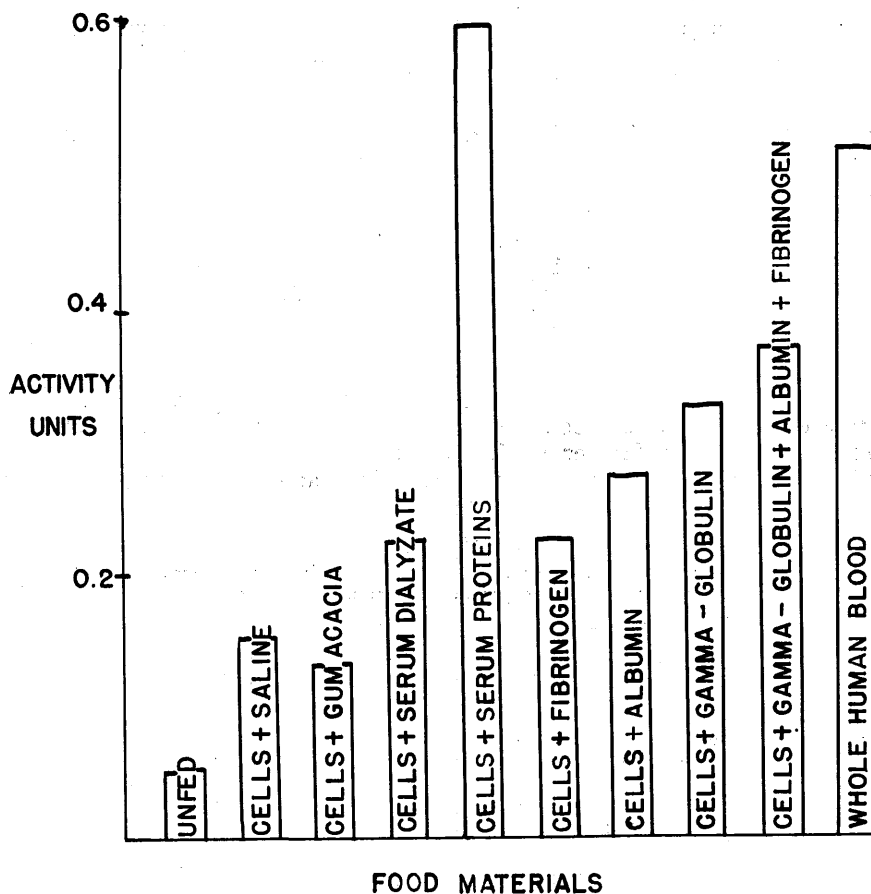


FIGURE 1. Relative amounts of protease activity in adult *Aedes aegypti* females in response to various food materials eighteen hours after feeding.

suspended in physiological saline to find the enhancement due to the cellular proteins in an inert medium. There was a slight increase in activity following the addition of the cellular proteins. Gum acacia was mixed with erythrocytes and was fed to discover any stimulatory effect due to viscosity or midgut distention. There was none. The addition of the prepared non-proteinaceous serum dialyzate increased the protease activity one and one-half times. When non-dialyzable sheep serum proteins were fed, a significantly higher amount of activity was demonstrated than with whole human blood. Both were significantly higher than the other materials tested here.

Three bovine plasma protein fractions (albumin, fibrinogen, gamma-globulin) were fed separately in 0.6 percent solutions. Protease activity with fibrinogen was not different from that with serum dialyzate. Activity with albumin was slightly higher, whereas gamma-globulin increased enzyme activity substantially. Additivity in stimulatory effect by the plasma fractions was tested by feeding the mixture of three fractions with erythrocytes. Enzymatic response to this mixture was greater than to any one fraction, but was not greater than the summed effect of the separate fractions (in excess of the value for erythrocytes alone). This is shown in figure 1.

*Relation of Activity to Amount of Blood Ingested.* Table 2 shows the quantitative effect of human blood on protease activity. The weight of blood per midgut is calculated from the weight of ingested blood divided by the number of mosquitoes fed that amount and not by the number of midguts in the final solution. These data were analyzed by the bivariate correlation coefficient test. The calculated

TABLE 2

*The effect of the weight of whole blood on the protease activity in adult Aedes aegypti females.*

WT. BLOOD INGESTED (mg.)	PROTEASE ACTIVITY (Opt. Dens.)	NO. OF MIDGUTS*	ACTIVITY PER MIDGUT	WT. BLOOD PER MIDGUT	WT. BLOOD PER ACTIVITY UNIT (Opt. Dens.)
1.10	0.044	0.77	0.057	0.28	4.9
2.18	0.050	0.77	0.065	0.55	8.5
2.85	0.148	0.77	0.192	0.71	3.7
3.43	0.205	0.77	0.267	0.86	3.2
4.75	0.272	0.77	0.353	1.19	3.4
6.93	0.385	0.96	0.401	1.38	3.4
7.01	0.453	1.73	0.262	0.77	2.9
7.25	0.417	0.77	0.542	1.81	3.3
8.01	0.389	0.77	0.505	2.01	4.0
8.30	0.500	1.35	0.370	1.18	3.2
8.31	0.399	0.77	0.518	2.08	4.0
9.64	0.490	0.77	0.636	2.41	3.8
10.95	0.417	0.58	0.718	3.65	5.1
11.03	0.466	0.77	0.605	2.76	4.6

\*Number of midguts in the final solution.

correlation coefficient of 0.944 indicates a strong positive interaction between the weight of blood ingested by a mosquito and its subsequent protease activity. This rectilinear relationship is shown in figure 2. This interaction remains whether a small number of mosquitoes was fed large volumes of blood or a large number of mosquitoes was fed small amounts of blood.

A set of three tests was made to test for the presence of a possible zymogen-kinase relationship in these digestive enzymes. Nine mosquitoes were fed normally on the forearm and allowed to digest the meal for 18 hr. The amount of blood ingested was determined. A similar amount of blood was mixed with nine freshly homogenized midguts and incubated 18 hr. at 40° C while the same amount of blood was incubated and then mixed with nine freshly homogenized midguts. The modified small sample technique for enzyme analysis was run on these three tests. There was no significant difference between the boiled blanks and runs in any test except for the normally fed mosquitoes, indicating that no zymogen-kinase reaction exists *in vitro*.

*Results of Injected Series.* Unfed mosquitoes injected with a mixture of saline and insect blood from blood-fed mosquitoes showed no increase in enzyme activity 8 or 10 hr. after injection (optical density of the final solution, 0.014).

Enzyme analyses were not possible on unfed mosquitoes injected with a mixture saline and midgut contents of blood-fed mosquitoes since three to four hours after injection, many of the internal viscera including the midgut were digested, leaving the tracheae seemingly intact.

*Bases for Calculation of Dilution Factors and Activity Units.* Assuming the average volume and the average weight of midgut tissue per female if 0.03 cu. mm. and 0.03 mg., respectively (Fisk, 1950), the milligrams of female midgut tissue per milliliter and the ratio of the volume of midgut tissue to the final solution read in the colorimeter can be calculated. In the case of 20 midguts, these figures would be 0.0132 mg. and 1:20,831, respectively. More detailed computations are given by Shambaugh (1953).

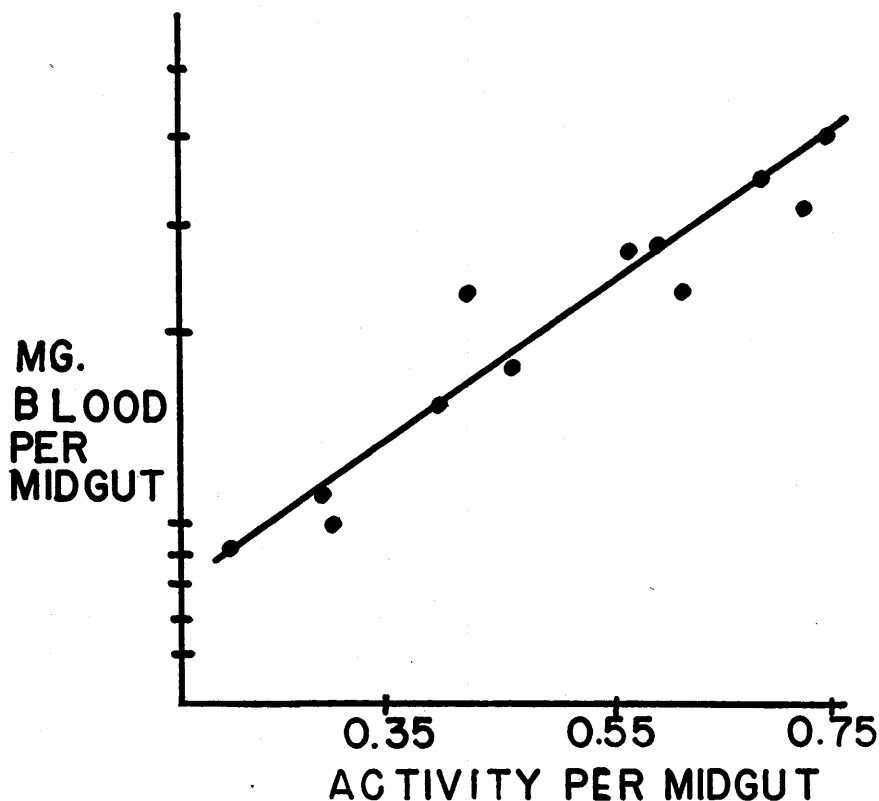


FIGURE 2. The ratio of the amount of blood ingested to the protease activity in adult *Aedes aegypti* females.

Protease activity may be expressed in terms of the velocity constant,  $K$ , of enzyme reactions:

$$K = \frac{1}{t} \times 2.3 \log. \frac{C_1}{C_2}$$

where  $C_1$  and  $C_2$  are the initial and final concentrations of protein substrate respectively after  $t$  minutes of digestion. The ratio of substrate concentrations can be derived from the optical densities as previously stated.

The initial substrate concentration ( $C_1$ ) would correspond to a 1:3 dilution of the stock solution of the azoprotein substrate. This calculated figure is 7.36.

The final substrate concentration ( $C_2$ ) is the difference between  $C_1$  and the optical density of each trichloroacetic acid filtrate. Enzyme activity of the original midgut solution may be obtained by multiplying the velocity constant by the total dilution factor of each solution (Charney and Tomarelli, 1947). The activity units (actually velocity constants) are arbitrary and are not to be confused with the Willstätter units for trypsin activity. In spite of the calculations required, these units have no more merit than the optical densities used to express protein concentration and thus protease activity. Therefore they have not been calculated here, but further examples are given by Shambaugh (1953).

#### DISCUSSION

The mere presence of an enzyme in the digestive tract of an insect does not irrevocably establish its substrate as a food of the particular insect (Fraenkel, 1940), although in insects the correlation between food and enzymes to digest them is good. Perhaps the correlation of these enzymes with their actual substrates can be made more evident by a more thorough research program on enzyme specificity and activators. Yet MacGregor (1931) reported that male mosquitoes, force-fed by the "capillary-tube" technique of unsheathing the fascicle, were capable of digesting blood, normally never taken by males, within the same period of time required by females. This technique was tried, but the author was unsuccessful in his attempt to induce either sex of mosquitoes to ingest blood in this manner.

Although Day and Powning (1949) observed that all enzymes were stimulated irrespective of the food given to American and German roaches (omnivorous feeders), preliminary results with an intermittent feeder on a restricted diet, such as the female *Aedes aegypti* mosquito, indicate a differential stimulation of enzymes dependent on the food offered these insects (Fisk and Shambaugh, 1952; Fisk and Shambaugh, in press).

The mechanism responsible for stimulation may be either hormonal or secretagogue (due to food or food products). Histological evidence of an increase in regenerative cells in the midgut of *Tenebrio molitor* after the injection of blood from fed beetles into unfed beetles led to the conclusion that the enzyme stimulation in this species is hormonal (Day and Powning, 1949). Previous work and results in the above section indicate a secretagogue stimulation in *Aedes aegypti*.

To test for conceivable precursors or zymogens present in the midgut upon which an activator or kinase possibly present in the blood could act, unfed individuals were tested against blood-fed individuals as described in the previous section. The results indicate that no zymogen-kinase relationship with blood exists. Fisk (1950) reported that no combination of crops, salivary glands, and empty midguts showed any measurable increase in protease activity. However, his tests were run after only two hours incubation.

Since there was no evidence for activation by endocrine inducement nor for a zymogen-kinase relationship, one can consider the effect of the various fractions of blood on protease activity as given in table 1. The residual value is given for unfed or blood-starved individuals. The effect of cellular proteins in an inert medium (erythrocytes in saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a dialyzable, weak, stimulatory factor in the plasma of blood. The non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified the protease activity to a level four times that found for erythrocytes and saline, indicating a strong, stimulatory effect. There was no difference between the digestive enzyme activities of those mosquitoes fed whole human blood and those fed whole sheep blood.

Inasmuch as the non-dialyzable (mostly protein) fraction of the plasma stimulated the most activity, various fractions of plasma proteins were fed separately and, once, collectively. Fibrinogen excited an elaboration of enzymes comparable to that of the plasma dialyzate. Plasma albumin increased the enzyme activity more than fibrinogen but not as much as gamma-globulin. The mixture of the three 0.6 percent solutions of proteins and erythrocytes stimulated more activity than any of the fractions alone. However, this amount of activity was less than that of the sum of the differences of the enzyme activities of each protein fraction minus the value for cellular proteins. This discrepancy is probably due to an error in sampling (only one test was possible), or is derivable from the effect of having the concentration of proteins in the composite substitute solution different than in the separate solution.

There exists a strong interaction between the amount of blood ingested by female mosquitoes and subsequent protease activity. The calculated correlation coefficient was 0.944, indicating that the blood ingested serves to increase the amount of protein substrate for the midgut proteases.

In the past two decades much valuable information has been accumulated for the understanding of the physiology and infectivity of malarial parasites through the use of bird malarial parasites and culicine mosquitoes. MacGregor (1931) reported that avian malarial parasites failed to develop if ingested into the diverticula. This fact would suggest that some reaction in the midgut, perhaps some process of digestion, was necessary for development. Huff (1927, 1934) concluded that immunity was not explained by differences in digestion processes between resistant and susceptible strains of mosquitoes discernible by histological methods. Other than genetic variation, the degree of infectivity and oöcyst development are greatly influenced by temperature and not by humidity nor activity nor age of the mosquito (Huff, 1941 a, b).

The number of oöcysts developed in the mosquito midgut is not directly correlated to the number of infective cells in the meal. This would indicate factors limiting infectivity other than exposure. In an infected mosquito the greater the number of oöcysts, the greater the number of eggs laid, but an increase in the number of eggs laid does not necessarily increase oöcyst formation. This was interpreted to be correlated with the absorption of blood after digestion (Hovanitz, 1947). Eyles (1952a) was able to demonstrate that serum alone was sufficient for full development of the parasite, but one-quarter development could occur on erythrocytes in saline. The dialyzable portion of the plasma had no effect. Bovine plasma albumin promoted mosquito infection in concentrations less than six percent when compared with physiological saline alone, but infections were less intense than those resulting from defibrinated blood.

There are still undetermined factors in the vertebrate blood affecting infection. Transfusion of normal blood into highly parasitized chickens was found to enhance the infectiousness of the blood in two or three attempts. Size of oöcysts, as well as their number, was found to be affected by the intensity of infection in the chicken (Cantrell and Jordan, 1946; Eyles, 1952 b).

Physiological studies on the blood of malaria-infected persons show increases in the blood of cellular proteins (after cellular disintegration), fibrinogen, and euglobulin, with an accompanying decrease in plasma albumin. Since plasma proteins have been shown to affect both protease stimulation and malarial infectivity, a strong interaction between these processes, formerly discounted, is suggested.

#### SUMMARY

1. Results of previous studies which indicated that the stimulation of proteolytic enzyme in the midgut of *Aedes aegypti* is secretagogue in nature served as the basis for this research. The technique of feeding adult female mosquitoes blood substitute solutions through warmed membranes is described.



2. A series of mosquito injection tests supports the previously proposed secretagogue theory of protease stimulation. Tests for conceivable zymogens present in the midgut stimulated by a kinase carried in the ingested blood (similar to the trypsinogen-enterokinase relationship of vertebrates) were negative. Various blood fractions were tested for secretagogue influences. In order for blood substitute solutions to be dispatched primarily to the midgut of this species of mosquito where protease elaboration is confined, it was expedient to mix them with thrice-washed erythrocytes.

3. A residual value of protease activity is given for unfed, or blood-starved individuals. The effect of cellular proteins in a relatively inert medium (sheep erythrocytes in physiological saline) is somewhat greater than the residual value. Both the dialyzable and non-dialyzable portions of blood were tested. The dialyzable portion mixed with erythrocytes was one and one-half times as effective in enzyme stimulation as the mixture of erythrocytes in saline. This would indicate a dialyzable stimulatory factor in blood plasma. However, the non-dialyzable plasma proteins and erythrocytes resuspended in saline intensified enzyme activity to an amount four times that of erythrocytes in saline. Evidently the more important stimulatory factors are in the non-dialyzable plasma proteins.

4. The effect of three plasma protein fractions at a concentration of 0.6 percent was tested. Fraction I (fibrinogen), normally 6 percent of plasma proteins, stimulated a secretion of enzymes comparable to that of the plasma dialyzate. Fraction V (albumin), normally 48 percent of plasma proteins, provided an increase in activity above that of fibrinogen. Fraction II (gamma-globulin) normally 6 percent of plasma proteins, stimulated a secretion greater than the sum of the fractions I and V. Enzymatic response to the mixture of these three plasma proteins was greater than to any one fraction, but was not greater than the summed effect of the separate fractions (in excess of the value for erythrocytes alone).

5. Through a series of experiments utilizing individual weights it was possible to discover a positive correlation between the amount of blood ingested by female mosquitoes and the subsequent protease activity of their midguts. This would lead to the conclusion that an increase in ingested blood increases the amount of the protein for the midgut proteases.

6. Discussions of the effect of blood and blood fractions fed to mosquitoes upon the degree of infectivity with malarial parasites are given. Since plasma proteins have been shown to affect both protease stimulation and malarial infectivity, a strong interaction between these processes, formerly discounted, is suggested.

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